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Polystyrene Beads Coated with Antibodies Directed to HLA Class I Intracytoplasmic Domain: The Use in Quantitative Measurement of Peptide-HLA Class I Binding by Flow Cytometry

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ABSTRACT: Protein-reactive, conformation-independent anti-peptide antibodies were raised in rabbits against a C-terminal sequence SDSAQGSDVSLA, common to most HLA-A and -B locus products. Antibodies were coupled to 4.5-μm polystyrene beads through the Fc portion by the use of protein A. The antibody-coupled beads showed a high capacity to bind HLA-A and -B proteins as well as their α chains by the intracytoplasmic domain, keeping the extracellular domains solvent exposed. The density of HLA class I proteins bound on the beads was approximately the same as that on cultured B cells. The antibody beads made it possible to quantitate peptide-HLA class I binding, *i.e.*, *in vitro* HLA class I assembly by flow cytometry. The assembly rate determined by the provisionally called flow cytometric HLA class I assay was 15%–19% for the reassembly of dissociated HLA class I proteins with the released selfpeptides. With single synthetic peptides, the highest rate so far obtained was 6.5%. The assay specificity and reproducibility were satisfactory. *Human Immunology 61, 1298–1306 (2000).* © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: HLA class I; polystyrene beads; flow cytometry

ABBREVIATIONS

APC	antigen-presenting cell
MFI	mean fluorescence intensity
TFA	trifluoro acetic acid
EDC	ethyl-dimethylaminopropyl-carbodoiimmide
CTL	cytotoxic T lymphocyte

INTRODUCTION

In the last 20 years, protein-reative antipeptide antibodies have been generated against a number of cellular components involved in the innate and acquired immune responses and used widely in the immunochemical detection and identification and the structural and functional characterization of those components. Antipeptide antibodies against HLA class I proteins were first produced in rabbits against a synthetic peptide derived from the first extracellular domain of HLA-B7 α chain sequence [1]. The antibodies were reactive with denatured HLA class I α chains but not with native HLA class I proteins. Subsequently, a synthetic peptide derived from the intracytoplasmic domain of HLA-B7 α chain sequence was used [2]. The corresponding antibodies were found to bind both native and denatured HLA class I α chains, conforming to the current view that antibodies raised against N- or C-terminal peptides that are linear, solvent-exposed, hydrophilic and flexible have a high probability to be conformation-independent and to bind the native proteins from which those peptides are derived [3].

Antipeptide antibodies directed to the C-terminal segment of HLA class I α chains could be ideal reagents for an oriented coupling of HLA class I proteins, because

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TABLE 1 HLA class I intracytoplasmic domain peptides

Peptide	Amino acid position	Sequence
A/B	326–338	C-S-D-S-A-Q-G-S-D-V-S-L-T-A
C	324–338	C-A-S-S-N-S-A-Q-G-S-D-E-S-L-I-A
A	311–323	C-K-S-S-D-R-K-G-G-S-Y-S-Q-A

they should not induce structural or conformational alternations of the HLA class I extracellular domains, keeping intact their antigen presentation capability as well as their allotypic specificity. Accordingly, in the present, work rabbit antibodies directed to a C-terminal peptide common to most HLA-A and -B α chains were produced and suitably coupled through their Fc portion to polystyrene beads. The beads were characterized for HLA class I binding activity and then tested for the utility in quantitative determination of peptide-HLA class I binding. The use of po lystyrene beads made it possible to analyze peptide-HLA class I binding by flow cytometry.

MATERIALS AND METHODS

Peptides

Three peptides deriving from the intracytoplasmic domain of HLA-A, -B, and -C α chains (Table 1) were synthesized by the conventional solid-phase method, using the Fmoc approach by aid of a Vega (Phoenix, Arizona, USA) semiautomatic peptide synthesizer Model 1000. A cysteine residue was added at the N-terminal end of each peptide to facilitate the subsequent preparation of KLH (Keyhole limpet hemocyanin)-peptide conjugates for immunization and also of Sepharose-peptide conjugates for antibody purification. Synthesized peptides were cleaved and fully deprotected with a mixture of TFA, anisole, and mercaptoethanol at a ratio of 19:1:0.5, precipitated with cold ether and then purified by ion-exchange column chromatograhy or reverse-phase HPLC, or both. Each preparation was verified for the amino acid composition.

Antibody Production and Purification

Purified peptides were conjugated to KLH through the N-terminal cysteine residue with MBS (m-maleimidobenzoyl-N-hydroxysuccnimide ester; Pierce, Rockford, IL, USA). Rabbits were immunized first subcutaneously with 300 μg of KLH-peptide conjugate in complete Freund adjuvant and then with 200 μg of the conjugate in incomplete Freund adjuvant several times at 10-day intervals.

Antipeptide antibodies were purified specifically by affinity chromatography on peptide-coupled Sepharose. Peptides were coupled to Sepharose-EAH (Pharmacia, Piscataway, NJ, USA) through the N-terminal cysteine residue with MBS. Peptide-coupled Sepharose was packed into a small column (0.5 × 4 cm) and washed with 0.2 M glycine-HCl buffer (pH 2.6) and then with PBS. Crude immunoglobulin fraction (20 to 30 mg) precipitated from antisera with a one-third saturation of ammonium sulfate was loaded on the peptide-coupled Sepharose column. The bound fraction was eluted with 0.2 M glycine-HCl buffer (pH 2.6), immediately neutralized, and then dialysed against PBS.

Peptide ELISA

A microwell plate (96-well, Falcon 3911; Becton Dickinson, Mountain View, CA, USA) was coated with test peptide in PBS (20 μg/ml, 50 μl/well) for 1 h, treated with 5% dry milk/0.2% Tween 20/PBS (200 μl/well) for 1 h, and then allowed to react with purified rabbit antipeptide antibodies at a serial threefold dilution for 1 h, all at room temperature. Bound antibodies were detected by the use of horse radish peroxidase coupled goat anti-rabbit IgG antibodies (50 μl/well, 2000 × diluted; Sigma A0545, St. Louis, MO, USA) and soluble substrate OPD (0.4 mg/ml, 50 μl/well) in 50 mM citrate-phosphate buffer (pH 5.0) containing 0.01% H_2O_2. The reaction was terminated with 2N H_2SO_4 (50 μl/well) and the color intensity was measured at 490 nm by a microwell plate reader.

HLA Class I ELISA

A microwell plate (96-well, Falcon 3911) was coated with mouse anti-HLA class I frame work antibody W6/32 in PBS (20 μg/ml, 50 μl/well) overnight in the cold and blocked with 5% dry milk/0.2% Tween 20/PBS (200 μl/well) for 1 h at room temperature. The plate was then allowed to react with test specimens (50 μl/well) at a serial threefold dilution with 0.02% BSA/0.1% Tween 20/PBS for 1 h and then with purified antipeptide antibodies (10 μg/ml, 50 μl/well) or with anti-HLA class I antiserum R6096 (50 μl/well, 200 × diluted) in 5% dry milk/PBS for 1 h, both at room temperature. Bound antibodies were detected as described above for peptide ELISA.

HLA Class I α Chain Preparation

HLA class I α chain-rich fractions were isolated from alkaline-denatured B-cell lysates by gel filtration according to a modification of the method previously described for HLA class I refolding assay [4]. In brief, cultured B cells (50 × 10^6 cells) were lysed at 4°C by the use of 1% Triton X-114 [5]. The X-114-bound membrane components were condensed at 32°C for 10 min and then dissolved with 0.5% Renex-30 (0.5 ml). The sample was

denatured at ~pH 11.7 by addition of 1 N NaOH and gel-filtered on a column (10 × 170 mm) of Sephadex G75-superfine. The first protein peak was located by the ninhydrin reaction on filter paper. The main fractions were combined and used.

Protein A-Coupled Polystyrene Beads

One-half milliliter of a 2.5% suspension of 4.5 μm carboxylated polystyrene beads (Polysciences, Warrington, PA, USA) was washed with 20 mM sodium phosphate buffer (pH 4.5) and resuspended in 0.6 ml of the same phosphate buffer. The bead suspension was incubated with an equal volume of a 2% EDC solution for 3 h at room temperature. The carboniimide-treated beads were washed with pH 4.5 phosphate buffer and resuspended in 0.6 ml of 200 mM borate buffer (pH 8.5) containing 200 μg of Protein A (Sigma). After overnight incubation at room temperature, the Protein A-coupled beads were treated with 0.1 M ethanolamine to block Protein A-unreacted sites and with 1% BSA to block nonspecific protein binding sites and then resuspended in 1 ml of 1% BSA/5% glycerol/0.1% NaN_3/PBS.

Anti-HLA Class I Peptide Antibody-Coupled Polystyrene Beads

A 1.25% suspension (100 μl) of Protein A-coupled polystyrene beads were allowed to bind with purified anti-peptide antibodies (20 μg) for 1 h and then with normal rabbit serum (100 μl) for 30 min at room temperature. After washing with 0.1% BSA/PBS, beads were resuspended with 100 μl of 0.1% BSA/PBS.

Biotinylated Anti-HLA Class I Antibody Reagents

Purified anti-HLA class I frame work antibody W6/32 and IgG fraction of rabbit HLA class I α chain antiserum R5996 [6] were biotinylated. Two milligrams of IgG were allowed to react with 75 μg of Sulfo-NHS-LC-biotin (Pierce) in 1 ml of 50-mM sodium bicarbonate buffer (pH 8.0) for 30 min at room temperature. Unreacted biotin was removed by dialysis against PBS. The binding specificity and activity of these biotinylated anti-HLA class I antibody reagents were shown in Table 2 as tested on intact and acid-treated cultured B cells by flow cytometry. A brief treatment of viable cells at acidic pH induces dissociation of cell surface HLA class I proteins and expose the α chains, not affecting cell viability.

Flow Cytometric HLA Class I Assay

Test samples (40 μl) were incubated with anti-HLA class I peptide antibody-coupled polystyrene beads (10 μl) overnight at 4°C. Beads were washed with 10% FCS/0.1% Renex 30/PBS and then with 10% FCS/PBS. Beads were incubated with a biotinylated antibody reagent (10 μl) for 1 h at room temperature, washed with 10% FCS/PBS, and then incubated with R-phycoerythrin-coupled streptavidin (150 × diluted, 50 μl; Caltag, Burlingame, CA, USA) for 30 min at room temperature. After washing with 10% FCS/PBS, beads were resuspended with 0.1% BSA/0.02% NaN_3/PBS and subjected to flow cytometry by FACScan.

TABLE 2 Binding activities of biotinylated HLA class I antibody reagents with HLA class I proteins expressed on cell surface

Biotinylated antibody reagents	Amounts μg/test	Mean fluorescence intensity	
		Intact BTB	Acid-treated BTB
W6/32	2	2173	205
	1	1811	180
	0.5	1291	131
R5996	3	301	1726
	1	151	888
	0.5	68	340
NRbt	1	5	5

BTB cells (0.5×10^6/test), untreated and acid-treated (pH 2.9, 2 min), were incubated with graded amounts of biotinylated antibody reagents and then with R-phycoerythrin-labelled streptavidin for 30 min, both at 4°C for 30 min. Stained cells were subjected to the flow cytometry by FACScan. Mean fluorescence intensity was determined at 495 V.

RESULTS AND DISCUSSION

Binding Activities of Rabbit Anti-HLA Peptide Antibodies With the Immunizing Peptides

Rabbit antibodies were raised against three peptides derived from the intracytoplasmic domain sequence of HLA-A, -B and -C α chains (see Table 1). Peptides A/B (326-338) and C (324-338) correspond to the C-terminal segment of HLA class I α chains. The former is shared by most A and B locus products, while the latter is common to the C locus products. These two peptides differ only in three positions. Peptide A (311-323) is derived from the intracytoplasmic segment next to the transmembrane region of HLA-A locus products. Antibodies were purified by affinity chromatography on peptide-coupled Sepharose and tested reciprocally for binding with peptides by the direct ELISA, using OPD as the substrate (see the materials and methods section). An unrelated peptide was included in the assay as the negative control. A_{490} (absorbance at 490 nm) was plotted against amounts of test antibody.

Both anti-A/B and anti-C bound peptide A/B (Figure 1). The former was higher, approximately three times, in the antibody titer than the latter. The amount of anti-A/B that induces one unit of A_{490} was 0.031 μg. Similar cross-reactivity was seen in the binding with peptide C, although the binding activity was much less than that with peptide A/B (data not shown). Anti-A was specific

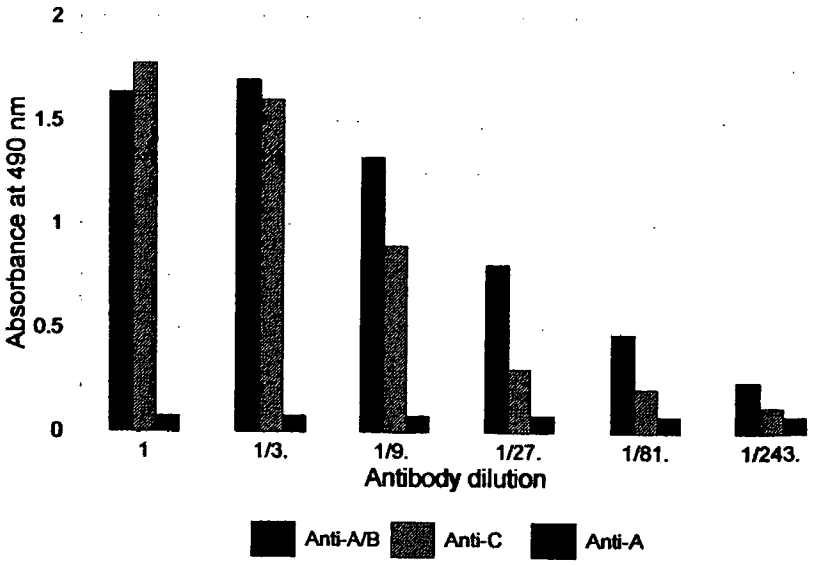

FIGURE 1 Binding activities of anti-HLA class I peptide antibodies with the immnizing peptides. Assay plates (96-well) were coated with test peptides (1 μg/well) and incubated with purified test antibodies (50 μl/well) at a serial threefold dilution starting from 10 μg/ml. The bound antibodies were detected by the use of HRP-anti-rabbit IgG antibodies and OPD (see the materials and methods section). Absorbance at 490 nm obtained for peptide A/B is shown as histogram.

to peptide A and no cross-reaction was seen with peptides A/B and C (data not shown).

Binding Activities of Anti-HLA Class I Peptide Antibodies With HLA Class I Proteins

Anti-peptide antibodies are not necessarily protein reactive. Accordingly, these anti-HLA class I peptide antibodies were assayed for binding with non-ionic detergent lysates of cultured B cells and a preparation of papain-solublized HLA-B7 protein [7] by the HLA class I ELISA (see the materials and methods section). As positive control, rabbit anti-HLA class I antiserum R6096 raised against papain-solubilized HLA-B7 protein [8] was used. A_{490} values were plotted against test sample dilutions (Figure 2). Anti-A/B bound well to BTB (A2, B27, Cw1) and C1R/B*2702 (B27) lysates (Figure 2a). A binding plateau was seen at ~0.7 of A_{490}. No significant binding was seen for lysates of HLA class I deficient cells, Daudi, C1R, and also with a preparation of papain-solubilized HLA-B7 protein that lacks the transmembrane and intracytoplasmic domains. Rabbit anti-HLA class I antiserum R6096 used as the positive control bound strongly to BTB and C1R/B*2702 lysates as well as to papain-solubilized HLA-B7 and weakly to C1R and T2 lysates (Figure 2b). No binding was seen for Daudi lysate. It is known that Daudi cells are negative for HLA class I, C1R cells positive for Cw4 [9] and T2 cells weakly positive for A2 and B51 [10].

These data indicate that anti-A/B antibodies are indeed protein reactive and do bind to the intracytoplasmic domain of HLA class I proteins. However, the binding activity does not seem to be sufficient to detect low levels of HLA class I expressed on C1R or T2 cells. Anti-A and anti-C were also tested for binding with BTB cell lysate and were found negative (data not shown). The lack of protein reactivity seen for anti-A is probably attributable to an insufficient local flexibility or exposure of the protein segment from which peptide A was derived. In the case of anti-C, the immunization may not be enough to produce the protein-reactive antibodies of high affinity. It is also possible that the protein segment from which peptide C was derived is conformationally different from peptide C.

Binding Activites of Anti-HLA Class I Peptide Antibodies Coupled to Polystyrene Beads

Anti-A/B antibodies were coupled to polystyrene beads by the use of Protein A (see the Materials and Methods section) and then tested for their binding with JY (A2, B7, Cw7) cell lysate and the HLA class I α chain-rich fraction (see the Materials and Methods section). Similarly, polystyrene beads carrying normal rabbit IgG (NRbt-beads) were prepared and tested as control. Bound proteins were detected by the flow cytometric HLA class I assay (see the materials and methods section), using biotinylated monoclonal antibody W6/32 reactive with the extracellular domains of all HLA class I proteins, biotinylated rabbit antibody R5996 specific to denatured HLA class I α chains and R-phycoerythrin-labeled streptavidin. Biotinylated normal rabbit IgG (NRbt) was used as negative control. Coupling of antibodies to polystyrene beads made it possible to analyze the antigen-antibody binding by the flow cytometry.

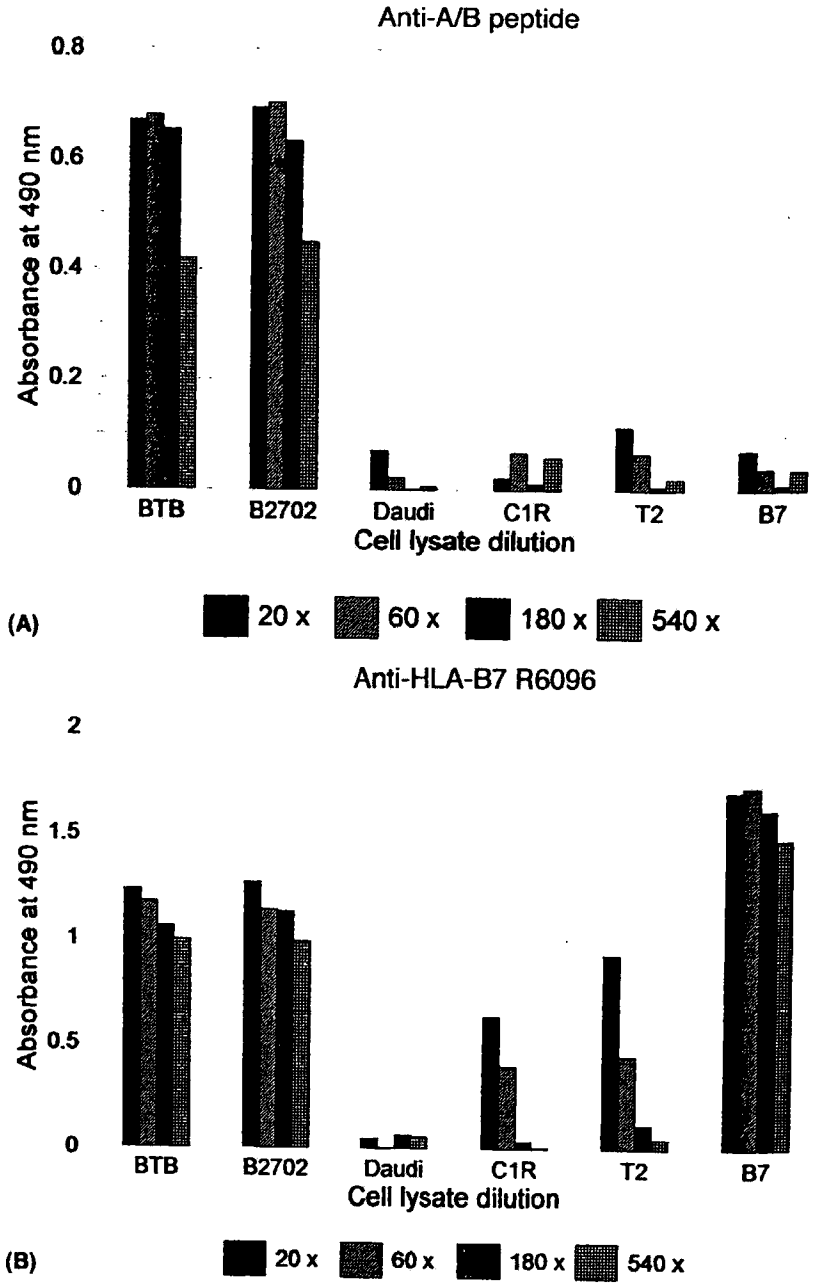

FIGURE 2 Binding activities of anti-HLA class I peptide antibodies with HLA class I proteins. Assay plates (96-well) were coated with anti-HLA class I frame work antibody w6/32 (1 μg/well) and incubated with test specimens (50 μl/well) at a serial threefold dilution starting from 20× dilution. After washing, plates were incubated with anti-A/B peptide antibodies (0.5 μg/well) or with rabbit anti-HLA class I antiserum R6096 (200× dilution, 50 μl/well). Bound antibodies were detected by the use of HRP-anti-rabbit IgG antibodies and OPD. Test specimens were the high-speed supernatants of 0.5% Renex 30 cell lysates (10^8 cells/ml) and a papain-solubilized HLA-B7 preparation. Absorbance at 490 nm obtained for anti-A/B peptide antibodies (A) and for anti-HLA class I antiserum R6096 (B) is depicted as histogram.

Results were given by the mean fluorescence intensity (MFI) determined at 620 V (Table 3).

Anti-A/B beads bound well HLA class I proteins as indicated by the high specific binding with W6/32. The MFI at antiboy excess (0.5–2 μg) was ~1100. Anti-A/B beads also bound HLA class I α chains as revealed by the reactivity with R5996. The specific binding was ~850 MFI.

The 1100 MFI at 620 V obtained for HLA class I proteins bound to anti-A/B beads is equivalent to MFI 200 at 495 V, because the fluorescence intensity measured at 620 V is 5.5 times greater than that measured at

TABLE 3 Binding activities of anti-A/B polystyrene beads with HLA class I proteins and the α chains

Biotinylated reagents	Amounts μg/test	Mean fluorescence intensity			
		Incubated with JY cell lysate		Incubated with JY α chains	
		Anti-A/B beads	NRbt beads	Anti-A/B beads	NRbt beads
W6/32	2	1138.2	69.5	11.5	14.7
	1	1121.5	69.5	10.9	13.3
	0.5	1169.9	68.8	9.8	12.4
R5996	3	651.4	168.2	1588.0	734.1
	1	522.2	118.6	1156.0	533.6
	0.33	384.9	79.1	545.2	318.4
NTbt	3	39.4	51.9	39.4	32.3
	1	29.5	40.7	29.5	26.0

Protein A-coupled polystyrene beads (a 1.25% suspension, 10 μl) were incubated with anti-A/B antibodies (2 μg) or normal rabbit IgG (2 μg) overnight at 4°C. Resulting anti-A/B and NRbt beads were incubated with 10 μl of JY (A2, B7, Cw7) lysate (10^8 cells/ml) or with 40 μl of the α chain-rich fraction (derived from ~10 μl of JY lysate) overnight at 4°C. Beads were then tested for binding with graded amounts of biotinylated W6/32 or R5996 or NRbt by the flow cytometric HLA class I assay (see Materials and Methods). Mean fluorescence intensity was measured at 620 V.

495 V. This MFI 200 is one-eleventh of MFI 2200 obtained for HLA class I proteins expressed on BTB cells (see Table 2). However, the diameter of the polystyrene beads used is 4.5 μm and that of cultured B cells is more than 15 μm, implying that the former has ~10 times less surface area than the latter. Therefore, the surface density of HLA class I proteins on anti-A/B beads is approximately the same as that on BTB cells. It could be even higher, because the surface of polystyrene beads is smooth, while that of B cells are known to be very rough.

HLA CLASS I ASSEMBLY WITH SELF-PEPTIDES IN THE PRESENCE OF ANTI-A/B POLYSTYRENE BEADS

The possible utilization of anti-A/B polystyrene beads as a solid immunoabsorbent in quantitative determination of peptide-HLA class I binding was evaluated in *in vitro* HLA class I assembly with self-peptides [11, 12] .

Nonionic detergent lysates of cultured B cells, JY (A2, B7, Cw7) and BOL (A2, B62, Cw10), were denatured by alkaline-treatment at pH 11.7. This treatment dissociates most HLA class I proteins into the α and β chains and releases the bound endogenous peptides. After neutralization, the denatured cell lysates were incubated with anti-A/B beads at 4°C. Sampling was made at Time 0 and at Days 1, 2, and 3. Each sample was incubated for 1 h at room temperature and then tested for binding with W6/32. Because *in vitro* HLA class I assembly takes place very slowly, Time 0 sample essentially stands for residual HLA class I proteins that are not dissociated by alkaline treatment, while Day 1, 2, and 3 samples include, in addition, HLA class I proteins assembled during the incubation period.

As shown in Table 4, in the case of JY lysate, HLA class I proteins detectable with W6/32 increased from 48.7 to 201.9 by 2-day incubation. This level remained much the same at Day 3. As to BOL lysate, the binding with W6/32 increased from 37.0 to 168.7 by 2-day incubation and then to 224.9 by 3-day incubation.

Because anti-A/B beads incubated with excess B-cell lysates give a MFI value of ~1100 in the binding with W6/32 (Table 3), it follows that HLA class I α chains re-associated with β_2-m and selfpeptides on anti-A/B beads account for 15% to 19% of HLA class I proteins that can bind to anti-A/B beads.

HLA Class I Assembly With Synthetic Peptides in the Presence of Anti-A/B Polystyrene Beads

HLA class I α chain-rich fraction was isolated from nonionic detergent cell lysate of each of four B-cell lines, BTB (A2, B27, Cw1), JES (A2, B27, Cw1), BOL (A2, B62, Cw10), and SPL (A31, B62, Cw1) by alkaline denaturation and gel-filtration (see the materials and methods section), and then incubated with β_2-m and known A2- and B62-binding peptides [13] in the pres-

TABLE 4 HLA class I assembly with self-peptides on anti-A/B polystyrene beads

Assay sample	Mean fluorescence intensity	
	JY cell lysate	BOL cell lysate
Time 0	48.7	37.0
Day 1	175.5	126.3
Day 2	201.9	168.2
Day 3	200.8	224.9

JY(A2, B7, Cw7) and BOL(A2, B62, Cw1) cell lysates (10^8 cells/ml) were denatured at an alkaline pH ~ 11.7 for 30 min. After neutralization, the lysates (10 μl/test) were incubated at 4°C in the presence of anti-A/B beads (a 1.25% suspension, 10 μl/test). HLA class I proteins bound to anti-A/B beads were determined at time 0, and incubation day 1, 2, and 3 with biotinylated W6/32 by the flow cytometric HLA class I assay. Mean fluorescence intensity was measured at 620 V.

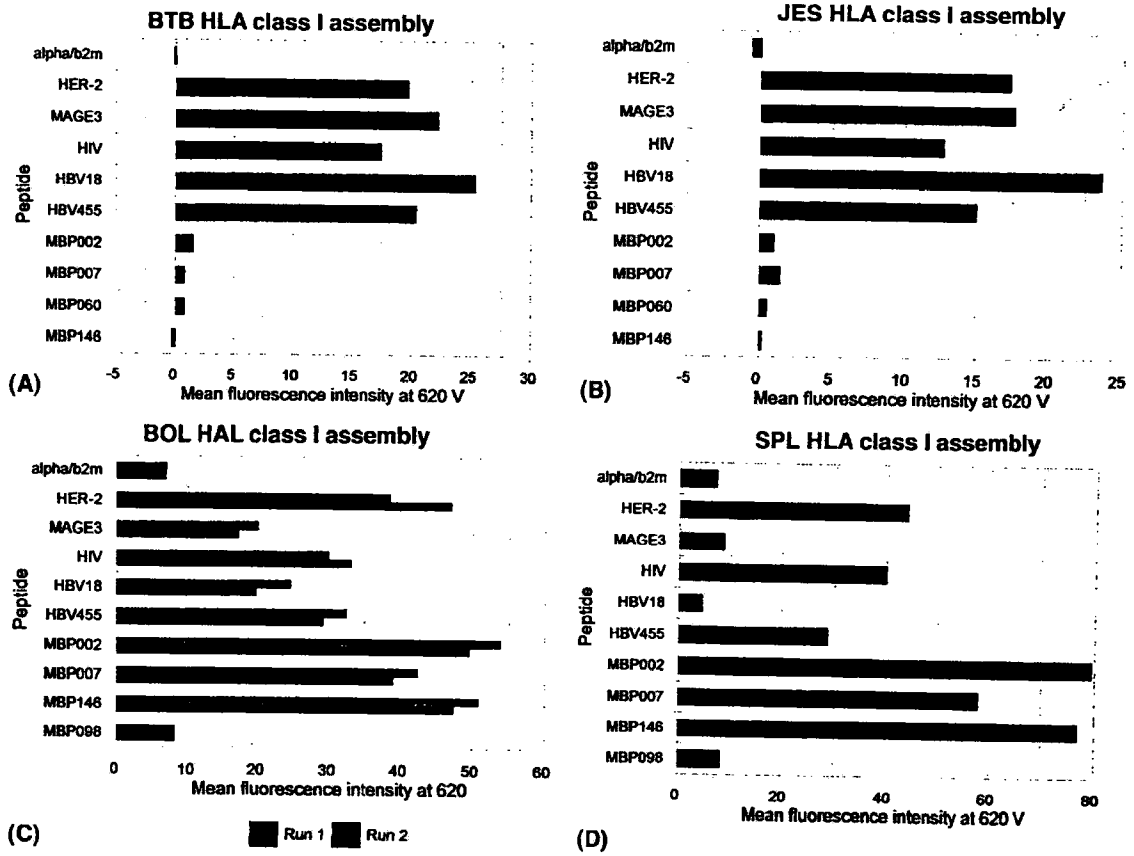

FIGURE 3 HLA class I assembly with synthetic peptides in the presence of anti-A/B polystyrene beads; BTB(A2, B27, Cw1), JES(A2, B27, Cw1), BOL (A2, B62, Cw10), and SPL (A31, B62, Cw1) α chains were isolated by the X-114 method (see the materials and methods section). Alpha chains (40 μl), test peptide (500 μg/ml, 5 μl), and β_2-m (100 μg/ml, 5 μl) were incubated in the presence of anti-A/B polystyrene beads (a 1.25% suspension, 10 μl) at 4°C for 2 days, and assayed for binding with W6/32 (1 μg) by the flow cytometric HLA class I assay (see the materials and methods section). Controls without peptide, *i.e.*, α chains alone and α chains plus β2m, were included in the assay. MFI was determined at 620 V. Values from which MFI obtained for α chains alone was subtracted are presented as histogram. The peptides tested: (1) A2-binding peptides were HER-2 (KIFGSLAFL), MAGE3 (FLWG PRALV), HIV (ILKEPVHGV), HBV18 (FLPSDYFPSV), and HBV455 (GLSRYVARL); (2) B62-binding peptides were MBP002 (SQKRPSQRH), MBP007 (SQRHGSKTL), MBP060 (SHHPARTAH), and MBP146 (AQGTLSKIF); and (3) the B51-binding peptide was MBP098 (TPPPSQGKG).

ence of anti-A/B beads for 2 days at 4 °C. Anti-A/B beads were harvested and assayed along with no peptide controls, *i.e.*, α chains only and α chains plus β_2-m for binding with W6/32 by the flow cytometry. MFI was determined at 620 V. The value obtained for α chains only was 6.1 for BTB, 12.1 for JES, 7.1 (Run 1) and 8.4 (Run 2) for BOL, and 10.5 for SPL. The net gain, *i.e.*, the MFI from which this control value was subtracted, is presented as histogram in Figure 3. This assay was carried out at a fixed peptide concentration, *i.e.*, ~50 μM. If needed, the HLA class I binding affinity of given peptides can be determined by testing them at several different graded concentrations.

In the presence of β_2-m, substantial HLA class I assembly was seen for BTB and JES with A2-binding peptides (Figure 3a and 3b), and for BOL and SPL with A2- and B62-binding peptides (Figure 3c and 3d). The assembly rate was the best with A2-binding peptide HBV18 for BTB and JES, giving MFI 23 and 25, respectively. In the case of BOL and SPL, the best assembly was seen with B62-binding peptide MBP002. MFI values were 47 (Run 1) and 54 (Run 2) for BOL and 72 for SPL. The reproducibility was fairly good as seen in the repeated assay of BOL and also in the two tests of BTB and JES, carrying the same HLA class I allotypes. These data also indicated that the MAGE3 and HBV18 pep-

tides tested are specific to A2, whereas the HER-2, HIV and HBV455 peptides tested are cross-reactive with A31 or B62, probably with the latter.

As seen in Figure 3a for BTB, HLA-A2 assembly with the A2-binding peptides tested gave MFI that ranged from 17 to 25. These values corresponded to 2.2%–3.3% of the total HLA class I proteins that can be loaded on anti-A/B beads. This density is comparable to that of HLA class I proteins on JY cells that can be loaded with A2-binding peptides. A small fraction of HLA class I proteins on cell surface is functionally empty, *i.e.*, peptide-free or peptide-replaceable. These empty proteins have been used for extracellular peptide-loading on APCs including cultured B cells. JY (A2, B7, Cw7) cells carry approximately 30,000 empty A2 molecules on the cell surface [14]. This accounts for ~1.7% of the total 1,800,000 HLA class I molecules expressed on the cell surface [15]. Hence HLA-A2 density on JY cell surface that could be maximally loaded with single peptides can not exceed this value 1.7%. A much higher density was obtained in the case of HLA-B62 assembly as seen for BOL and SPL (Figure 3c and 3d). The density amounted to 11.5%.

Flow cytometry has been used to determine HLA class I reassembly on acid-treated B cells [16, 17]. Mild acid treatment dissociates HLA class I proteins expressed on cell surface. The dissociated class I proteins can be reassembled with β_2-m and specific peptides and the reassembly class I poteins can be quantitated by flow cytometry, using fluorochrome-labeled antibodies or peptides. These flow cytometric peptide-HLA class I binding assay methods are technically simple and also time saving, because it does not require isolation of HLA class I α chains. However, they are not freely applicable to systematic analysis of *in vitro* HLA class I assembly, because the use of viable cells limits the experimental conditions. Artificial microspheres coated with antibodies directed to a C-terminal segment of HLA class I α chains facilitate not only oriented coupling of HLA class I proteins, but also evaluation of various nonphysiological conditions for peptide-HLA class I binding. Thus, the present methodology is useful for nonradioactive assay of peptide-HLA class I binding, but it is much more significant for preparation of HLA class I microspheres loaded with single synthetic peptides that are well-defined for the surface density.

In conclusion, polystyrene beads coated with antibodies against HLA class I intracytoplasmic domain are suitable for measuring peptide-HLA class I binding by means of flow cytometry and also for preparing HLA class I microspheres that have potential for functional studies of single peptide-loaded HLA class I molecules.

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